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TECHNICAL MANUSCRIPT 816

EFFECTS OF OXYGEN ON
AEROSOLIZED SERRATIA MARCESCENS

George E. Hess

MAY 1965

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UNITED STATES ARMY
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U.S. ARMY BIOLOGICAL LABORATORIES
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TECHNICAL MANUSCRIPT 216

EFFECTS OF OXYGEN ON AEROSOLIZED SERRATIA MARCESCENS

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Physical Sciences Division
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ABSTRACT

Suspensions of Serratia marcescens (ATCC strain 14041) in water were aerosolized in a rotating drum in the presence of various concentrations of oxygen. Colony-forming ability of aerosolized organisms was rapidly destroyed by contact with 0.25% or more oxygen at 40% relative humidity and 25 C, but was almost unimpaired for at least 5 hours in nitrogen containing not more than 10 ppm oxygen. Completely hydrated organisms were insensitive to oxygen at pressures up to 100 pounds per square inch for four hours. No loss in viability occurred in aerosols of washed cells in air at 97% relative humidity. It is proposed that dehydration of the aerosolized cell results in sensitization to lethal effects of oxygen but is not the primary cause of death. Mn^{++} , Co^{++} , glycerol, and thiourea enhanced the biological stability of aerosols in air. Numerous similarities between the effects of oxygen in this system and in systems using lyophilized or irradiated organisms or cell-free enzymes support the hypothesis that closely related mechanisms are involved.

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I. INTRODUCTION

Dunklin and Puck,¹ observing that the death rate of airborne bacteria varies with relative humidity (RH), suggested that the sensitivity of microorganisms to toxic agents increased at a critical degree of cellular dehydration. Webb² attributed lethal effects in the aerosol to collapse of protein structures upon dehydration and later stated³ that additives capable of replacing cellular water increased the survival of aerosolized bacteria. Zimmerman,⁴ however, proposed that nonpermeable carbohydrates enhance stability through a plasmolytic dehydration of the organism.

The tacit assumption, in most of this work, has been that dehydration, per se, causes the death of the cell by disrupting vital structures, concentrating toxic material, or imbalancing metabolic activity. Monk and McCaffrey⁵ showed that the death rate of rehydrated Serratia marcescens is maximal at a water content of 33%, but remarked that effects of O₂ on the death rate had not been determined.⁶ In this laboratory, studies of the effects of ascorbic acid on aerosolized S. marcescens suggested that interaction between the cells and atmospheric O₂ might contribute to the death of the cells. Preliminary experiments indicated that, when S. marcescens was aerosolized into air diluted with nitrogen, the death rate increased with O₂ concentration. The work described here was undertaken to test the possibility that drying sensitizes organisms to lethal effects of O₂, but is not the direct cause of death of aerosolized S. marcescens.

II. MATERIALS AND METHODS

S. marcescens (Fort Detrick, strain 8UK, ATCC strain 14041) was grown and stored as frozen pellets by a method already described.² Routinely, the thawed pellets were washed three times with distilled water, diluted to 20×10^8 viable cells per ml, shaken for 3 hours at 30 C, and stored at 4 C. When unwashed cells were required, pellets were thawed and diluted in water only. Thoroughly washed, heat-shocked Bacillus subtilis var. niger spores were added to the suspensions immediately before dissemination to achieve a final concentration of 2.0×10^8 viable spores per ml.

Samples for S. marcescens assays were diluted in gel saline solution (0.85% NaCl, 0.1% Bacto gelatin) with 0.005% potassium tellurite added to prevent growth of S. marcescens in the spore assays. Viable cell populations were determined by the standard surface plating technique on peptone agar, using six plates per assay.

B. subtilis spores showed complete biological stability in the aerosol regardless of RH or ambient gas composition. Therefore, the number of spores recovered from the aerosol indicated the maximum viable cell recovery (N_0) to be expected for S. marcescens in the same aerosol. Differences in control counts were corrected by the formula

$$N_0 = C_{SM} \cdot N_{BS} / C_{BS}$$

where C_{SM} and C_{BS} are the viable cell populations of S. marcescens and B. subtilis, respectively, in unaerosolized controls, and N_{BS} is the number of viable spores recovered from the aerosol. Data are reported as the ratio N/N_0 , where N is the number of viable S. marcescens cells recovered from the aerosol. This method of calculating recovery minimized variables associated with dissemination and collection procedures and physical fallout from the aerosol (approximately 0.8% per min linear decay rate). Although appreciable batch-to-batch variation in absolute viable cell recovery levels was observed, a uniform data pattern was obtained in three sets of trials. This pattern is best illustrated by the data from one typical set of trials, which are presented below.

Aerosols were generated in a 86.6-liter stainless steel drum revolving at 5 rpm. The RH was measured before and after aerosol dissemination with an electric hygrometer element* held at the center of the drum. The element was shielded from the aerosol except during the time required to take readings. When the atmosphere was to contain oxygen at a preselected concentration, the system was flushed for at least 30 minutes with gas of about the

* HygroDynamics, Inc., Silver Spring, Md.

desired final composition. Toward the end of this period, either N₂ or air was added until the oxygen analyzer* indicated the proper oxygen concentration. No difference in results was observed when tank oxygen or tank air (breathing quality) was substituted for the laboratory supply or when argon was substituted for nitrogen. The RH was adjusted during the final stages of this procedure by passing the gas through either Drierite or water until an RH 5% lower than the preselected value was attained. Addition of aerosol to the system changed both the RH and gas composition, so final values were determined after the aerosol had equilibrated for 2 minutes.

When oxygen-free conditions were sought, the apparatus was evacuated to about 10⁻³ torr with a two-stage vacuum pump. This pressure was maintained for at least 30 minutes, then sufficient prepurified N₂** was added to bring the system to atmospheric pressure. Further purification of N₂ with pyrogallol-KOH or Fieser's reagent³ was not acceptable because of evolution of CO and H₂S from the two solutions, respectively, both of which seriously affect the aerosol stability of *S. marcescens****. The pumping and filling procedure was repeated three times; finally, the N₂ was passed through degassed water until the drum was filled with gas at a positive pressure of 2 cm Hg and at the desired RH.

Aerosols were disseminated for 60 seconds from a Vaponefrin standard nebulizer**** with a flow rate of approximately 0.25 gram of liquid in 5 liters of gas per min at a driving pressure of 400 mm Hg. The driving gas was either air or prepurified N₂, depending on the desired final composition of the drum atmosphere. The atomizer was inserted into a large-bore stopcock attached to the drum axle, permitting its removal without admitting air to the system. In experiments requiring the absence of oxygen, the atomizer and cell suspension were degassed after insertion into the system. This procedure was shown to exert no effect on the aerosol characteristics in air, and, when eliminated from the technique, resulted in only a small decrease in stability in nitrogen. Aerosols were collected for 30 sec in M.S.A. midjet impingers***** at a flow rate of 5 liters per min. The collection fluid consisted of 5 ml gel saline with 0.001% Antifoam A. The aerosol age was recorded as the interval between the midpoints of the dissemination and collection periods.

* Model 777, Beckman Instruments, Inc., Fullerton, Calif.

** Matheson Co., Inc., East Rutherford, N.J.; maximum O₂ conc = 8 ppm.

*** Unpublished data.

**** Vaponefrin Co., Edison, N.J.

***** Mine Safety Appliances Co., Pittsburgh 8, Pa.

III. RESULTS

Ratios between the numbers of viable S. marcescens and B. subtilis spores collected from aerosols stored in various concentrations of oxygen are shown in Figure 1. Maximum survival of S. marcescens occurred at the minimum oxygen concentration attained, and at this point was nearly equivalent to spore survival. As the oxygen concentration was increased, loss in viability increased, so that $\log N/N_0 = k \log P/P_0 + C$ (Fig. 2). All aerosols in this series were generated from suspensions of thoroughly washed S. marcescens free of added solutes. The tests were performed at 40% RH because aerosols of this organism routinely yield minimum survival in air at this humidity.

Lethal effects were observed when O_2 was added to aerosols originally disseminated into N_2 (Fig. 3). Although only 30% loss in viability occurred in nitrogen for as long as 5 hours (Table 1), the addition of as little as 5% oxygen resulted in at least 80% loss of viability within 30 minutes. This effect was noticed at oxygen concentrations as low as 0.25% and became greater as oxygen concentration increased. The addition of 5% oxygen after aerosols had been stored in nitrogen for up to 40 min was selected as an arbitrary example of this system.

Unwashed, unshaken S. marcescens were more sensitive to storage as aerosols in air (Table 1) and respired 5 to 10 times faster than washed, shaken cells. Stability in nitrogen was unimpaired, however, indicating a relationship between respiration and sensitivity to oxygen. This is consistent with our earlier observation, described by Goodlow and Leonard,⁹ that minimum survival in air is obtained with actively growing S. marcescens.

Effects of several additives on the stability of aerosolized S. marcescens are shown in Table 2. These compounds were selected because of their demonstrated influence on the stability of enzymes or organisms exposed to oxygen in other systems. The concentration of each compound inducing optimum stability in the aerosol, with no toxic effect on control organisms, was determined empirically. Cobalt and manganese compounds, as well as glycerol and thiourea, were protective for airborne organisms; cysteine, $CuSO_4$, and $MgSO_4$ were less effective. N-ethylmaleimide (NEM) was slightly protective in the presence of air but induced some sensitivity under anoxic conditions.

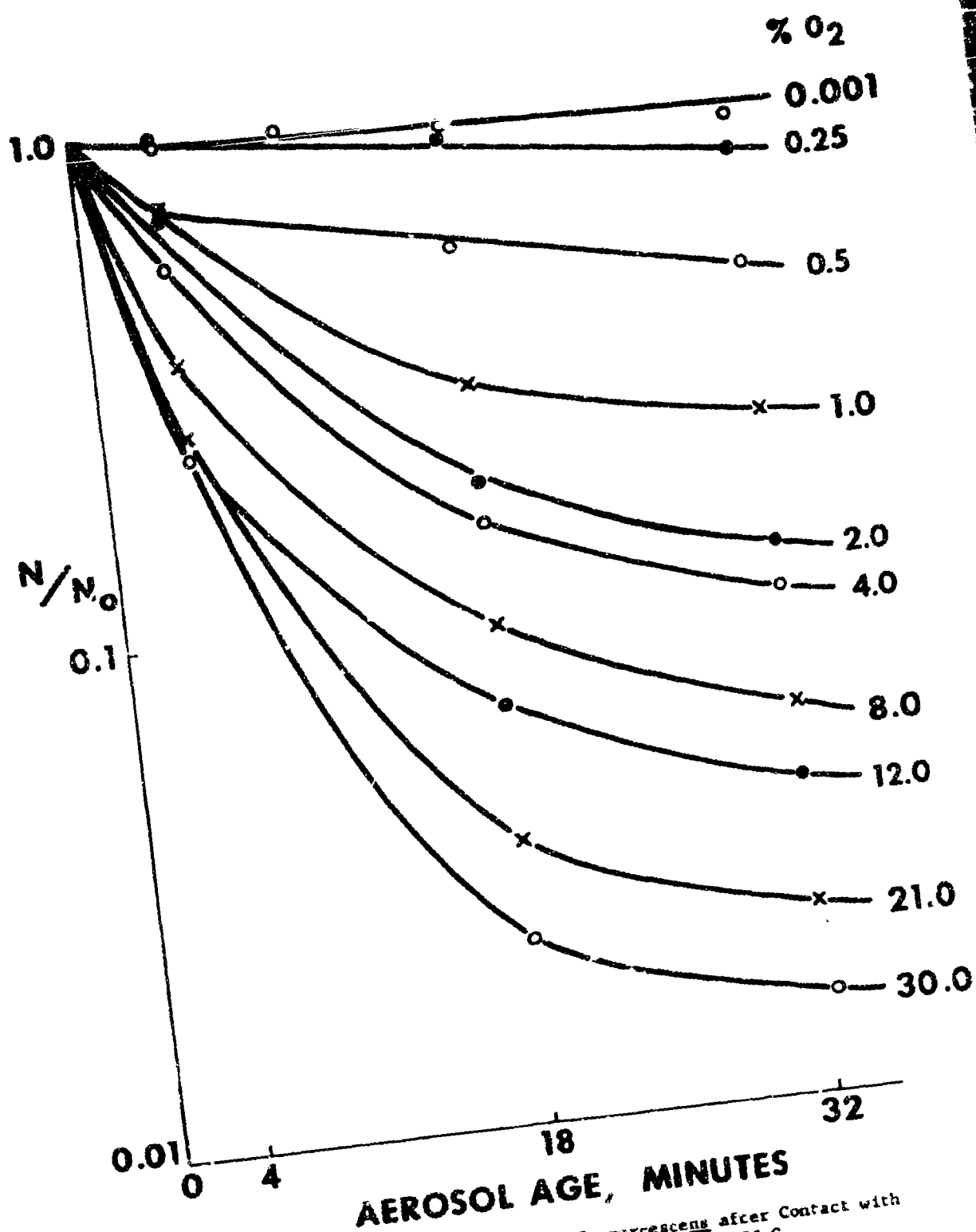


Figure 1. Survival of Aerosolized *S. marcescens* after Contact with Various Concentrations of O_2 at 40% RH; 25 C.

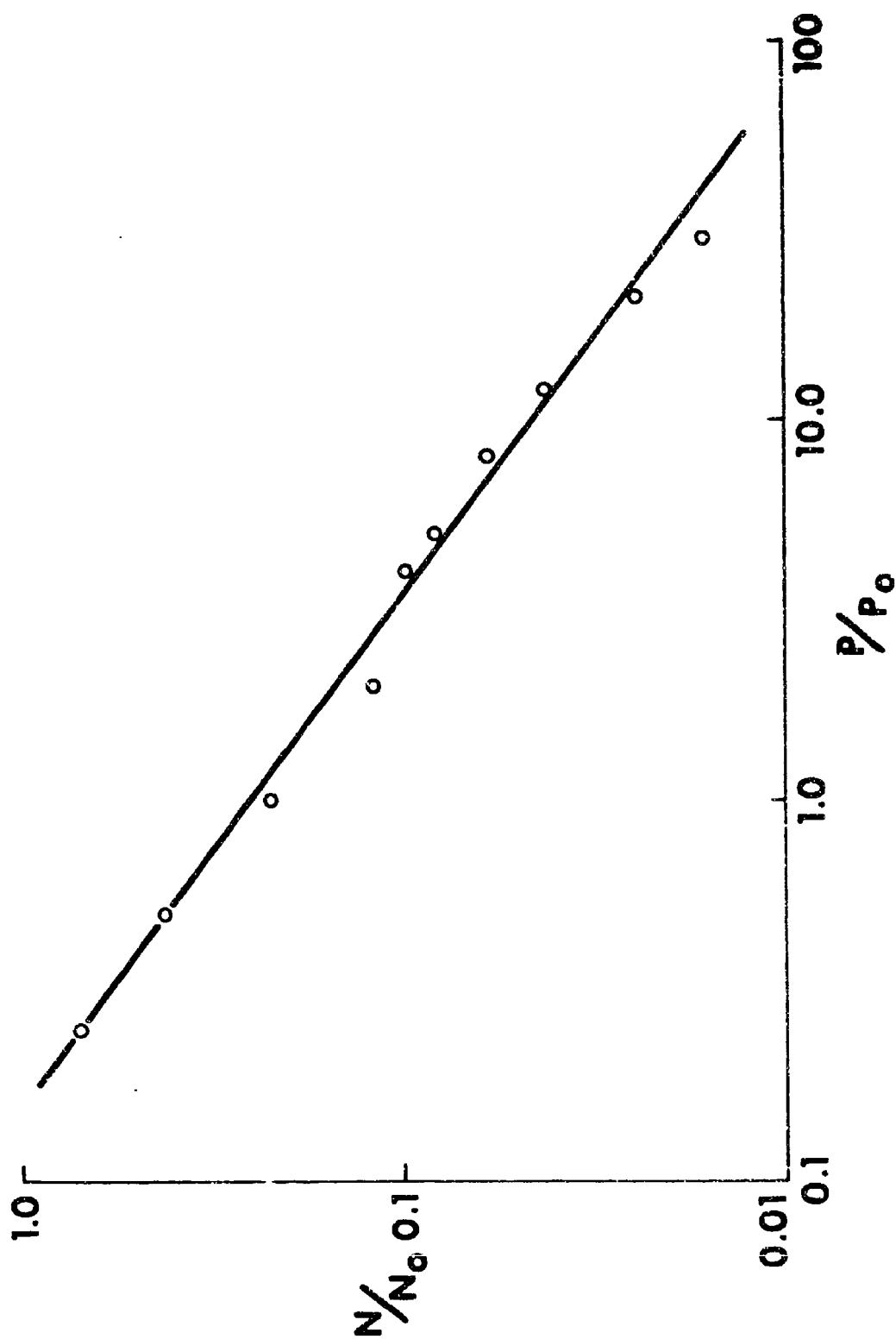


Figure 2. Log Log Plot of the Survival of Aerosolized Washed S. marcescens after 12 min vs. O_2 Concentration.

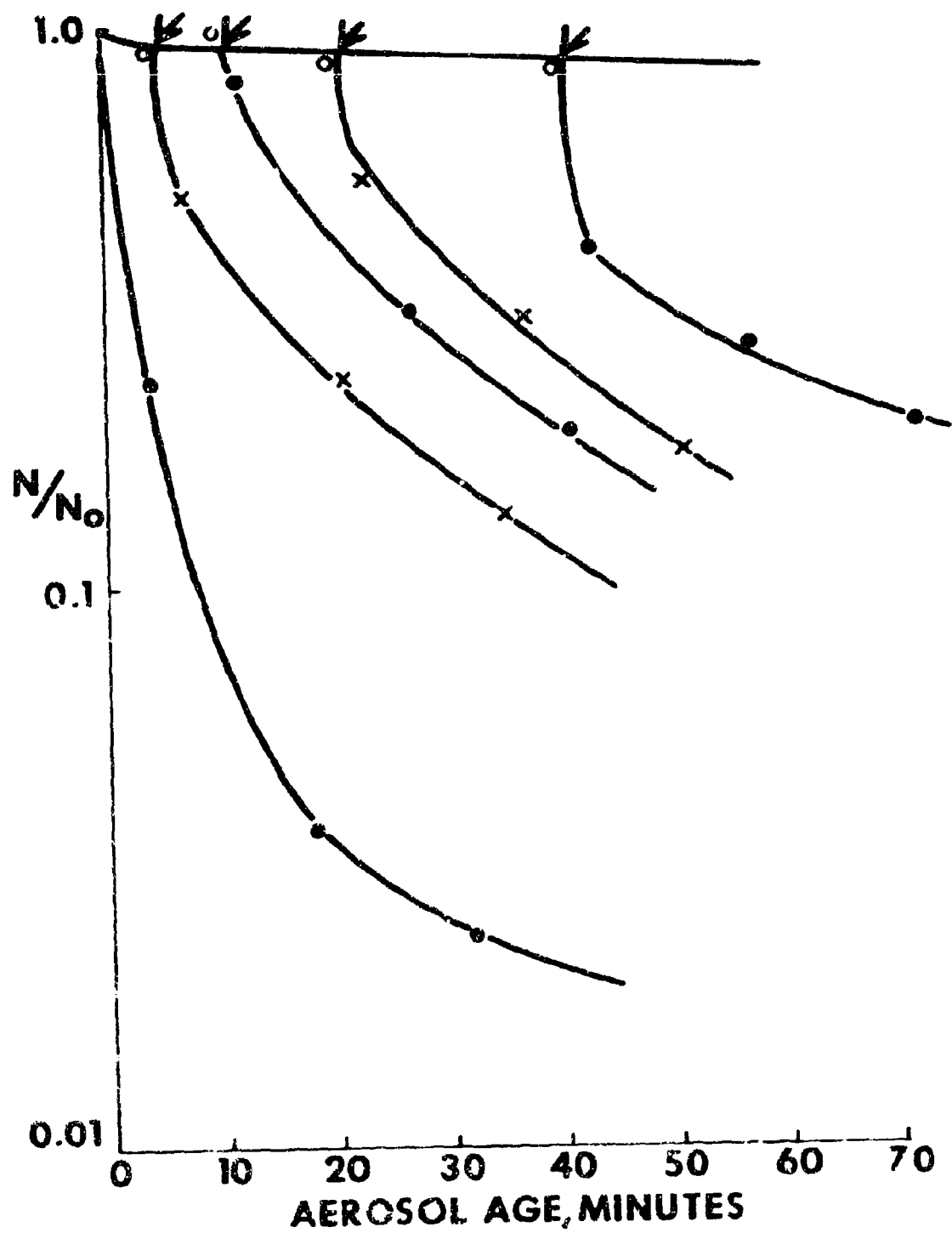


Figure 3. Survival of Washed *S. marcescens* Aerosolized in Nitrogen with 5% O_2 Added at Times Indicated by Arrows.

TABLE 1. SURVIVAL OF WASHED AND UNWASHED S. MARCESCENS AEROSOLIZED INTO AIR OR N₂^{a/}

Aerosol Age, minutes	N/N ₀ ^{b/}			
	Air		N ₂	
	Unwashed	Washed	Unwashed	Washed
4	0.009	0.230	1.0	1.0
18	0.0007	0.040	0.92	0.95
32	0.0001	0.024	0.90	0.90
150	Too low to assay	0.007	0.72	0.70
300	Too low to assay	0.004	0.70	0.69

- a. Suspensions contained 20×10^8 S. marcescens cells per ml and 2×10^8 E. subtilis var. niger spores per ml. All aerosols generated at 40% RH, 25 C.
- b. Ratio of S. marcescens to E. subtilis spores in the same aerosol, corrected for differences in control counts.

Aerosols of S. marcescens in air or nitrogen were collected, concentrated by centrifugation, and incubated in Bacto nutrient broth at 30 C. Un-aerosolized controls indicated that this organism is 0.5 to 0.75 micron in length in the resting state and normally divides before a maximum length of 5 microns is attained in log-phase cultures. Cells in cultures generated from air aerosols, however, showed three distinctly different morphologies after the lag phase had ended. A number of organisms, roughly corresponding to the expected viable population, elongated and divided normally. A second group, presumably dead cells, did not change in size, and gradually lost optical density as incubation progressed. The remaining cells were unusual, however, in that they elongated without signs of division, attaining lengths of 15 to 30 microns. Several of these giant cells were observed to burst under the phase microscope.

TABLE 2. EFFECTS OF VARIOUS COMPOUNDS ON SURVIVAL OF AEROSOLIZED S. MARCESCENS^{a/}

Compound	Concentration, moles/liter	N/N ₀ ^{b/} Aerosol Age, minutes		
		4	18	32
None (Air 97% RH)	-	1.07	1.03	1.00
None (Air 40% RH)	-	0.23	0.04	0.02
None (N ₂)	-	1.00	0.95	0.90
MnSO ₄	5 x 10 ⁻²	0.77	0.51	0.48
MnCl ₂	3 x 10 ⁻³	0.47	0.37	0.34
Mn(NO ₃) ₂	2 x 10 ⁻⁴	0.41	0.35	0.19
MgSO ₄	2 x 10 ⁻³	0.28	0.11	0.07
CoCl ₂	2 x 10 ⁻⁴ d/	0.52	0.22	0.13
NaCl	8 x 10 ⁻³	0.06	0.03	0.01
CuSO ₄	2.5 x 10 ⁻³ d/	0.20	0.04	0.03
Glycerol	1 x 10 ⁻²	0.47	0.28	0.18
Thiourea	1 x 10 ⁻²	0.44	0.25	0.21
Cysteine HCl ^{c/}	5 x 10 ⁻³	0.26	0.10	0.06
N-ethylmaleimide (air)	1 x 10 ⁻⁴ d/	0.20	0.07	0.05
N-ethylmaleimide (N ₂)	1 x 10 ⁻⁴ d/	0.75	0.44	0.40

- a. Suspensions contained 20 x 10⁸ S. marcescens cells per ml and 2 x 10⁹ B. subtilis var. niger spores per ml. All aerosols were generated in air at 40% RH, 25 C except as noted.
- b. Ratio of S. marcescens to B. subtilis spores in the same aerosol, corrected for differences in control counts.
- c. Brought to pH 7.0 with NaOH.
- d. Higher concentrations were toxic to control suspensions.

Experiments were performed to determine the sensitivity of completely hydrated S. marcescens to O_2 . Two-ml portions of cell suspensions containing 20×10^6 viable cells per ml were stored under O_2 at pressures from 15 to 100 pounds per square inch for 4 hours at 25 C. Both a static system and one in which gas continuously bubbled through the suspensions were used. No change in viability was observed, compared with controls under N_2 . Aerosols disseminated at 97% RH retained viability for at least 32 min (Table 2).

IV. DISCUSSION

No information is available on the extent of dehydration occurring in aerosolized washed microorganisms, but Bateman et al.¹⁰ described changes in water content of lyophilized S. marcescens as a function of aqueous vapor pressure. Although some hysteresis is apparent, essentially the same curves are shown for water sorption and desorption at 20 C. Approximately 85% of the original weight of sorbed water was lost from S. marcescens equilibrated at 40% RH, but only 10% or less was lost at 97% RH. It is assumed that a similar relationship between water loss and RH exists in the aerosols described here.

Aerosolized S. marcescens cells rapidly become non-viable in the presence of O₂ at 40% RH, but not at 97% RH. Similar aerosols are not inactivated in N₂ at either RH. Evidently some water loss from the organism must occur before lethal interaction with O₂ becomes possible. Furthermore, it appears that dehydration of aerosolized S. marcescens is not itself the primary cause of the death of the organism. Studies of the effects of O₂ on aerosolized microorganisms have apparently not been reported previously. Effects of O₂ in other biological systems are well known and are mentioned below as a possible guideline for future investigations into the basic mechanism involved in oxygen toxicity in the aerosol.

Stadie, Riggs, and Naugaard¹¹ reviewed the early literature describing effects of O₂ on enzymatic systems. They suggested that O₂ at ordinary pressures acts as an oxidizing agent to produce enzymatic inhibitions. A toxic action of O₂ on glucose and pyruvate oxidation in a cell-free multienzyme system has been observed.¹² Barron¹³ noted that pyruvic oxidase from gonococci is inactivated by exposure to gaseous O₂.

Dickens¹⁴ showed that brain tissue respiration was inhibited by O₂ and that Mn⁺⁺, Co⁺⁺, Mg⁺⁺, and Cu⁺⁺, in that order, decreased the extent of inhibition. He also observed that Mn⁺⁺, Co⁺⁺, and Mg⁺⁺ enhance pyruvic oxidase activity and suggested that the primary effect in oxygen poisoning of brain tissue may be through inhibition of this system. An alternate explanation of the protective effects of Co⁺⁺ and Mn⁺⁺ may be derived from the work of Gilbert, et al.,¹⁵ who reported that these ions decreased the net formation of H₂O₂ in glutathione solutions exposed to high O₂ pressures. Whether either of these mechanisms exists in aerosolized bacteria is not known, but it is interesting that relatively low concentrations of Co⁺⁺, Mn⁺⁺ tend to stabilize airborne S. marcescens (Table 2).

Data indicating that exposure to O_2 induces similar effects in aerosolized and lyophilized S. marcescens have been obtained in this laboratory, and are being prepared for publication. Contact between O_2 and lyophilized E. coli results in the death of the cells¹⁸ and the formation of free radicals.^{17,18} No method is available at present to collect sufficient aerosolized organisms after defined conditions of contact with O_2 for free radical determinations. Attempts to determine whether changes occur in nicotinamide adenine dinucleotide (NADH) oxidase activity in airborne bacteria have been unsuccessful, but Lion and Avi-Dor¹⁹ have shown that NADH oxidase activity is inhibited in lyophilized E. coli after exposure to O_2 .

Tallentire, Dickenson, and Collett²⁰ found that gamma-irradiated Bacillus megaterium spores were inactivated at the maximum rate when in equilibrium with water vapor at a pressure corresponding to 40% RH. Aerosolized S. marcescens cells are particularly unstable in air at this RH. Lethal effects of O_2 on irradiated organisms are well known,^{21,22} and Powers, Webb, and Ehret²³ suggested that reactions between free radicals and O_2 in irradiated dry spores resulted in toxicity. Thiourea and cysteine protect against radiation-induced mutation and inactivation of E. coli through processes not involving O_2 .²⁴ Thiourea, an excellent aerosol stabilizer, helps protect lyophilized E. coli against O_2 damage,²⁵ but cysteine is less effective in both systems.

Glycerol, in a class with thiourea and cysteine in irradiation studies,^{26,27} is an efficient aerosol stabilizer, but it is possible that this occurs because of effects on the loss of water from airborne organisms. Dewey,²⁸ however, noted that glycerol may act as a radical scavenger in irradiated S. marcescens. The effects of NEM on aerosolized S. marcescens are consistent with the results noted by Bridges²⁹ and Dewey,²⁸ using irradiated cells, in that sensitization occurred under anoxic conditions in both systems, but slight protection was observed in the presence of O_2 .

Perschman, et al.³⁰ noted that decreased metabolism has a protective effect in O_2 poisoning and possibly in X-irradiation systems also. They concluded that increased metabolism might result in an increased production of free radicals and thought it not surprising that variations in oxygen toxicity with metabolic activity have been found. This latter relationship is similar to that observed with airborne S. marcescens.

The filamentous growth of bacteria following X-irradiation,³¹ or irradiation,³² and other treatments is well documented. Filamentous growth of S. marcescens after exposure to air and incubation in nutrient has been noted, and recently Cox and Baldwin³³ reported similar results with E. coli. The mechanism responsible for this abnormal growth has not been investigated with airborne organisms, but Errera³⁴ and others have proposed that DNA synthesis is inhibited after irradiation so that normal division does not occur.

At present, only S. marcescens has been used in this study, but effects of O₂ on E. coli and several other organisms are being investigated and will be presented later. It is not proposed here that the mechanisms causing death of irradiated, lyophilized, or aerosolized organisms in the presence of O₂ are identical, but only that there are many similarities among them. Berechman et al.⁸⁰ hypothesized a common mechanism between O₂ poisoning and the initial effects of X-irradiation in biological systems. I propose that O₂ poisoning and death of airborne bacteria may be similarly related. The concept that cell death in the presence of O₂ in the four systems mentioned occurs for closely related reasons has helped me to explain and predict the behavior of aerosolized S. marcescens. This was found to be difficult or impossible via the hypothesis that dehydration alone caused the death of airborne microorganisms.

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army Biological Laboratories Fort Detrick, Frederick, Maryland, 21701
13. ABSTRACT	
<p>Suspensions of <u>Serratia marcescens</u> (ATCC strain 14041) in water were aerosolized in a rotating drum in the presence of various concentrations of oxygen. Colony-forming ability of aerosolized organisms was rapidly destroyed by contact with 0.25% or more oxygen at 40% relative humidity and 25 C, but was almost unimpaired for at least 5 hours in nitrogen containing not more than 10 ppm oxygen. Completely hydrated organisms were insensitive to oxygen at pressures up to 100 pounds per square inch for four hours. No loss in viability occurred in aerosols of washed cells in air at 97% relative humidity. It is proposed that dehydration of the aerosolized cell results in sensitization to lethal effects of oxygen but is not the primary cause of death. Mn^{++}, Co^{++}, glycerol, and thiourea enhanced the biological stability of aerosols in air. Numerous similarities between the effects of oxygen in this system and in systems using lyophilized or irradiated organisms or cell-free enzymes support the hypothesis that closely related mechanisms are involved.</p>	

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